

High Pressure Matrix-Assisted Laser Desorption/Ionization Fourier Transform Mass Spectrometry for Minimization of Ganglioside Fragmentation

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Transiently elevating pressure in a matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS) source into the 1–10 mbar range during ionization decreases the metastable fragmentation of gangliosides. This allows detection of the molecular ion species without loss of the highly labile sialic acid residues. In these experiments, gangliosides with up to five sialic acids were ionized by MALDI and detected with the FTMS. In each case, when the high pressure collisional cooling was used, the singly charged molecular ion was the base peak in the spectra, both in the positive and negative ion modes, and minimal metastable fragmentation was observed. This result is promising, as the previously developed TLC separation methods can be coupled to MALDI-FTMS. (J Am Soc Mass Spectrom 2002, 13, 402–407) © 2002 American Society for Mass Spectrometry

Gangliosides constitute a class of glycolipids that occur in the outer leaflet of the plasma membrane in all eukaryotic cells [1–3]. Gangliosides bear one or more *N*-acyl neuraminic acid (sialic acid) residues as branches on their carbohydrate moiety, an oligosaccharide with a linear backbone that is linked to a ceramide which may have heterogeneity in both its long-chain base and *N*-linked fatty acyl group [4, 5]. The long-chain base (LCB) generally has 18 or 20 carbons, the *O*-linked glycan is at C1 and there is a 3-hydroxyl group; the *N*-acylated amino group is in the 2-position, and unsaturation is usually present at C4, C5. Further hydroxylation and/or unsaturation of the LCB may also occur. The length of the fatty acyl group may vary from C16 to C26 or more, and it may be modified by one or more hydroxyl groups and points of unsaturation. Gangliosides play important roles at the cell surface, serving as antigens and/or receptors, e.g., for adhesion

[6, 7], attachment of extracellular matrix components, viruses, bacteria and toxins [8, 9], and as an important means for cell–cell communication [3, 10]. The variations in their structures are especially important during nervous system development and carcinogenesis [2, 11–13]. Like most glycoconjugates, gangliosides usually occur as mixtures of structural variants whose specific biological activity at any given time may be based on minor, transient components in the mixture. It is often not possible to obtain enough pure material for NMR structural determinations of the critical epitopes, and even when larger amounts of sample may be available, the progress of the isolation must be monitored by techniques with high specificity and sensitivity [2, 9, 14]. Due to its high sensitivity and capacity for dealing with complex mixtures, mass spectrometry often plays a critical role in the detection and structural determination of gangliosides.

Because the glycosidic bonds linking the sialic acid residues are especially labile, mass spectral analysis of gangliosides has always presented a particularly difficult challenge. The development of soft ionization techniques for mass spectrometry has greatly aided the ability to profile gangliosides present in biological tissues and follow the changes in their compositions [11, 12, 15–19]. The ability to ionize without fragmentation and with high detection sensitivity has begun to permit tandem mass spectrometric investigation of the detailed structures of gangliosides, even in unseparated mixtures [15, 19–26]. However, characterization of sialy-

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Abbreviations: Gangliosides are abbreviated according to the system of L. Svennerholm [1–3] as follows: G_{M1} , Gal β 1 \rightarrow 3GalNAc β 1 \rightarrow 4(Neu5NAc α 2 \rightarrow 3) Gal β 1 \rightarrow 4Glc β 1 \rightarrow 1'Cer; G_{D1a} , Neu5NAc α 2 \rightarrow 3Gal β 1 \rightarrow 3GalNAc β 1 \rightarrow 4(Neu5NAc α 2 \rightarrow 3)Gal β 1 \rightarrow 4Glc β 1 \rightarrow 1'Cer; G_{T1b} , Neu5NAc α 2 \rightarrow 3Gal β 1 \rightarrow 3GalNAc β 1 \rightarrow 4(Neu5NAc α 2 \rightarrow 8Neu5NAc α 2 \rightarrow 3) Gal β 1 \rightarrow 4Glc β 1 \rightarrow 1'Cer; G_{Q1b} , Neu5NAc α 2 \rightarrow 8Neu5NAc α 2 \rightarrow 3Gal β 1 \rightarrow 3 GalNAc β 1 \rightarrow 4 (Neu5NAc α 2 \rightarrow 8Neu5NAc α 2 \rightarrow 3)Gal β 1 \rightarrow 4Glc β 1 \rightarrow 1'Cer; Q_{P1} (in this case is a mixture of Q_{P1b} and Q_{P1c}), Q_{P1b} , Neu5NAc α 2 \rightarrow 8Neu5NAc α 2 \rightarrow 8 Neu5NAc α 2 \rightarrow 3 Gal β 1 \rightarrow 3GalNAc β 1 \rightarrow 4(Neu5NAc α 2 \rightarrow 8Neu5NAc α 2 \rightarrow 3) Gal β 1 \rightarrow 4Glc β 1 \rightarrow 1'Cer and Q_{P1c} , Neu5NAc α 2 \rightarrow 8Neu5NAc α 2 \rightarrow 3Gal β 1 \rightarrow 3 GalNAc β 1 \rightarrow 4 (Neu5NAc α 2 \rightarrow 8 Neu5NAc α 2 \rightarrow 3)Gal β 1 \rightarrow 4 Glc β 1 \rightarrow 1'Cer.

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lated species still represents one of the most difficult analytical undertakings in glycobiology and improved methods for their ionization are continuously being sought in order to maximize ionization efficiency and minimize the potential for decomposition during analysis to thereby increase the reliability and sensitivity of the procedures. In this contribution, we report on the use of a new high pressure MALDI source [27] for collisional cooling and stabilization of ganglioside molecular species during mass spectral analysis with Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS or FTMS).

Previous MALDI mass spectral work with gangliosides clearly showed that the fragile nature of the sialic acid linkages is a limiting factor for their analysis [16, 28–31]. With both Electrospray and MALDI mass spectrometry, the loss of sialic acid residues is the predominant fragmentation pathway. This property is particularly important for MALDI-FTMS where the relatively long time between ionization and detection (0.3–30 s depending on the resolution desired and the pumping efficiency of the specific vacuum system) allows unimolecular metastable fragmentation reactions to go to completion [28, 30, 32]. However, some methods have been developed to partially stabilize the gangliosides on desorption. In particular, cool matrices like 6-azo-2-thiothymine (ATT) [16] and 2-5-dihydroxybenzoic acid [33], having the property of decreasing the observed metastable decay in reflectron mode, were introduced in MALDI time of flight (TOF). Similarly, use of larger alkali cations [30], such as cesium, has been successful in reducing (but not eliminating) fragmentation of the gangliosides. Conversion of the carboxyl group to a methyl ester improves stability [18], as does permethylation [16], but derivatization is not an option when a bioassay is part of the overall characterization scheme.

Recently, work on MALDI at higher pressures, from 1–10 mbar [34] to 1 bar [35], has demonstrated that the hot MALDI ions can be cooled by desorption in a high pressure bath gas, and recent developments have extended this finding to FTMS at 10^{-2} mbar [36] and at 1–10 mbar [27]. In this study, the recently reported high pressure MALDI-FTMS ion source is investigated with respect to the analysis of these highly labile gangliosides containing up to five sialic acid residues.

Experimental

The instrument and new ion source were previously described in detail [27]. The ganglioside samples were prepared using ATT ($\sim 2 \mu\text{L}$ saturated in MeOH) as a matrix, and deposited onto the probe tip using Gel-Loader tips (Eppendorf, Brinkmann Instrument, Inc. Westbury, NY) to form a solid layer of matrix, and ~ 1 pmol of sample (in $1 \mu\text{L}$ 50:50 chloroform:isopropanol) was deposited on top of the matrix layer. As appropriate, $1 \mu\text{L}$ of 20 mM sodium acetate (NaAc) or cesium acetate (CsAc) was placed on the matrix layer and allowed to dry prior to addition of sample. Most

samples in this study were ganglioside standards purchased from Sigma (St. Louis, MO) with the exception of the G_{Q1b} and $\text{G}_{\text{P1b,1c}}$ samples, which were extracted from human brain tissue [37, 38]. Although some initial results [27] have demonstrated low femtomole–high attomole detection limits for small peptides, precise detection limits with this source have not been determined for gangliosides analysis.

The pulse sequence described previously is used without modification [27]. Internal calibration [39] is employed to achieve high mass accuracy on these spectra of known ganglioside standards with the most abundant peak (and its isotopes) and the lower homolog (and its isotopes) used for calibration.

Results and Discussion

Metastable activation of MALDI ions is a general phenomenon that applies to all analytes, but as most polymers and unmodified peptides are stable relative to the thermal activation energy deposited during MALDI, it is not generally investigated. However, metastable fragmentation becomes a problem when the analytes, like the gangliosides shown here, are very fragile, or when the analytes are mixtures of fragile molecules.

The utility of mass spectral analysis of gangliosides by MALDI-FTMS has been demonstrated, but its application has been hampered by extensive metastable fragmentation when standard MALDI sources are employed [27, 28, 30, 40, 41], and derivatization, e.g., permethylation, is often necessary to stabilize the more highly labile species sufficiently to enable MS analysis. In the case of homogeneous standards, metastable fragmentation can be used to verify the details of the ganglioside structures, but for heterogeneous samples like those typically extracted from biological tissues, extensive fragmentation during the analysis makes determination of the composition of the ganglioside mixtures extremely difficult or impossible. In the mass spectral analysis of a mixture, if there is substantial fragmentation on ionization, one cannot ascertain whether a particular ion is formed from an independent species in the mixture or is merely a fragment of a higher molecular weight compound. Therefore, the ability to ionize the species with no (or minimal) fragmentation is of prime importance to the analysis of gangliosides.

For gangliosides, MALDI-FTMS spectra are usually dominated by the loss of sialic acid residues, particularly when the glycolipids are underivatized. A typical example of this is presented in Figure 1. Figure 1a shows G_{D1a} ions, with 10 mM NaAc added to the solution prior to sample preparation, measured from the conventional MALDI-FTMS ion source. The reader should note that, in the labeling of the peaks in all the spectra shown in this and other figures, a form of shorthand notation is used to designate the charge-neutral exchange of H for Na or Cs, $\text{M}(\text{nNa})$ corre-

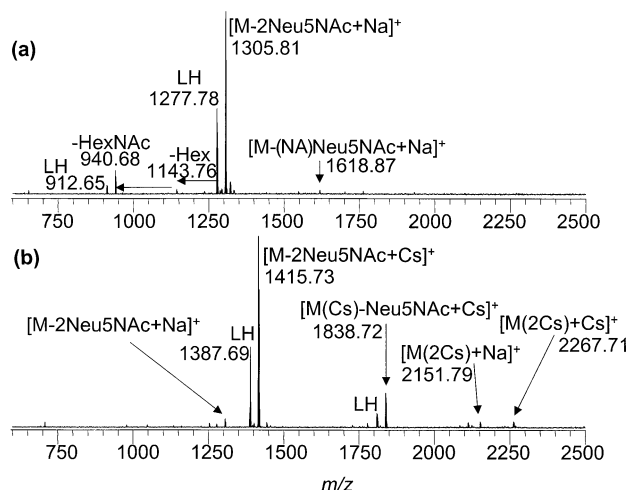


Figure 1. Positive-ion MALDI-FTMS spectra of G_{D1a} ionized on the conventional MALDI-FTMS source with (a) sodium and (b) cesium as the cation.

sponds to $M + nNa - nH$ [21]. The most abundant peaks are the ions that result from the loss of both sialic acid residues of the more abundant ganglioside and its lower homolog (LH) differing in mass by C_2H_4 due to heterogeneity in the ceramide moiety. A low abundance species is apparent in which only one of the two available sialic acids has been eliminated and there are additional glycosidic fragmentations corresponding to the stepwise cleavage of the glycosidic bonds connecting the non-reducing end hexose residues (162.05 Da) and the adjacent HexNAc residues (203.08 Da), as well as the respective lower homologs of each of these. This same sample was reanalyzed with 10 mM CsCl added to promote generation of the cesium-cationized species, and the resulting spectrum is shown in Figure 1b. Again in this spectrum, the most abundant peaks, corresponding to each of the two homologs, result from the loss of both sialic acid residues. However, the species resulting from a single sialic acid loss are now prominent, the unfragmented molecular ion species with both sialic acids retained are visible, and there is minimal further fragmentation. Clearly Cs^+ cations have somewhat stabilized the molecular ion. This is consistent with previous reports [30], but the presence of so many ion types generated from the starting homolog pair bodes ill for analysis of mixtures of unknown species with variable numbers of sialic acid residues.

High pressure collisional thermalization of gangliosides is demonstrated in Figure 2. In this case, sodiated G_{M1} was measured using the new ion source [27] with (Figure 2b) and without (Figure 2a) the pulsed high pressure background gas. Without collisional cooling (Figure 2a), the base peak in the spectrum is the asialo fragment. Some further glycosidic cleavages are apparent, and the sialylated molecular ion is not visible above the noise. However, when pressure is pulsed into the 1–10 mbar region during desorption (Figure 2b), the base peak is now the unfragmented sodiated molecular

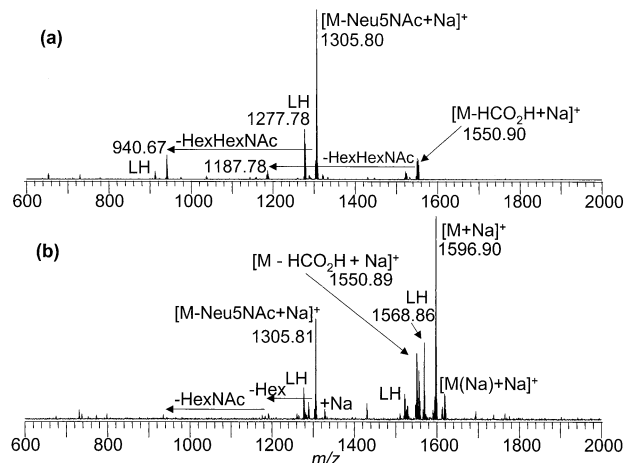


Figure 2. Positive-ion MALDI-FTMS spectra of G_{M1} ionized using the high pressure MALDI FTMS ion source (a) without and (b) with high pressure collisional cooling gas.

ion. Only a low abundance peak due to loss of sialic acid is still apparent, and the further losses of Hex and HexNAc are significantly reduced. Thus, these spectra illustrate clearly that collisional cooling leads to a decrease in fragmentation, since the asialo fragment at m/z 1305 is the most abundant ion in the absence of the gas, and the unfragmented molecular ion, m/z 1596, is the most abundant peak when the ions are desorbed at high pressure.

Similarly, for G_{D1a} (Figure 3a and b) and G_{T1b} (Figure 3c and d), the dominant peaks in the spectra shift from being those that correspond to the asialo fragment at m/z 1305 to those that correspond to the unfragmented sodiated molecular ions when the ions are desorbed at high pressure. The G_{D1a} spectrum (Figure 3b) includes several distinct contaminant peaks (marked with an asterisk) that correspond in mass to G_{T1} and its homolog. Such incomplete separation is common with species like these which have highly similar structures. Although G_{T1b} , or an isomer, is therefore obviously present in the G_{D1a} sample, these peaks are noticeably absent in the spectra obtained without collisional thermalization (Figure 3a) clearly illustrating the advantage of desorbing the unknowns at high pressure to avoid metastable decay in MALDI analysis of mixtures. Although at this point relative quantitation can only be approximated with such a sample because of the presence of residual sialic acid losses on ionization or in solution, combining the high pressure MALDI with the stabilization effect of using Cs cations would certainly minimize fragments and allow greater certainty on the relative concentrations of a mixture of species. Comparison of Figure 3a with Figure 1a, both spectra of G_{D1a} , shows that the new source [27] has generated a signal, albeit low abundance, for the molecular ion. As both spectra were obtained with threshold laser power, most likely the change in desorption geometry between the two sources has removed a mass filtering bias.

The human brain derived G_{Q1b} and $G_{P1b,1c}$ (Figure 4)

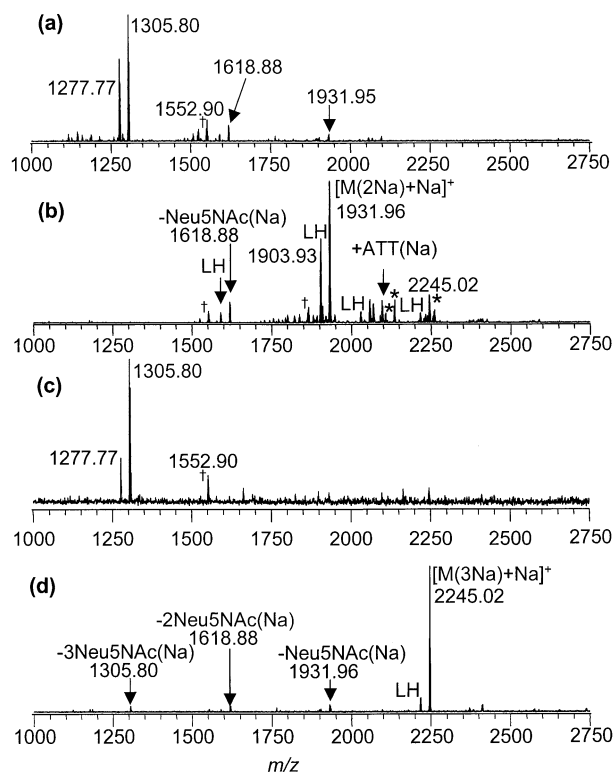


Figure 3. Positive-ion MALDI-FTMS spectra of G_{D1a} and G_{T1b} ionized using the high pressure MALDI FTMS ion source. (a) G_{D1a} without and (b) G_{D1a} with high pressure collisional cooling gas: (asterisk) indicates G_T contaminant peaks (c) G_{T1b} without and (d) G_{T1b} with high pressure collisional cooling gas. The peaks (dagger) at m/z 1865.9 and 1552.9 could arise respectively from decarboxylation of a species corresponding to $[M(2Na) + H]^+$ containing a protonated Neu5NAc and the subsequent loss of Neu5NAc(Na) from this decarboxylated ion.

samples also exhibit significantly reduced metastable fragmentation at high pressure. Although, as the number of sialic acid residues increases, the ion species involved become more fragile and hence, more prone to fragmentation by sialic acid loss and elimination of the

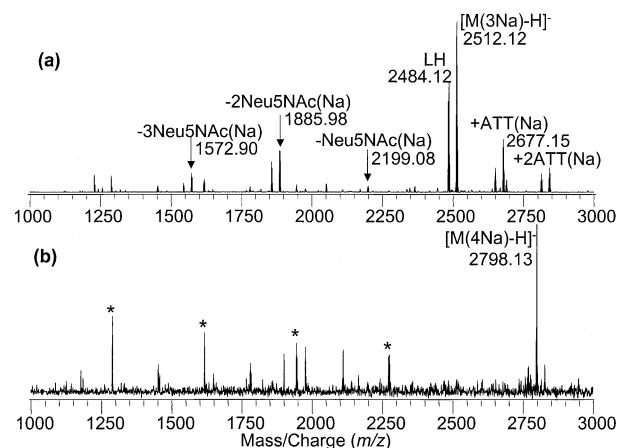


Figure 4. Negative-ion MALDI-FTMS spectra of (a) G_{Q1b} and (b) G_{P1} ionized using the high pressure collisional cooling gas with the MALDI-FTMS ion source. Asterisks denote matrix clusters.

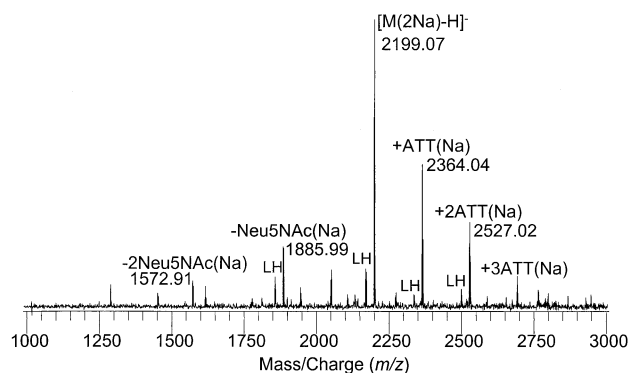


Figure 5. Negative-ion MALDI-FTMS spectrum showing adduction of matrix to G_{T1b} in the high pressure collisional cooling MALDI-FTMS ion source.

carboxyl group(s) as formic acid, in each case, with collisional cooling, the unfragmented sodiated molecular ion continues to be the base peak. These two spectra were obtained in the negative ion mode since for gangliosides negative ion mode is typically more sensitive, and the initial positive ion mode spectra showed weak (G_{Q1b}) or no ($G_{P1b,1c}$) signal even though (for G_{Q1b} at least) the same collisional cooling trend was noted. These two spectra represent a significant improvement over the only previous example of gangliosides of this size run by MALDI, in which extensive metastable decay resulted in mass spectra with a rolling baseline and seriously attenuated resolving power [16], even though these spectra were generated using a linear TOF in which post-source fragmentation would not be perceived as fragment ion peaks since product ions formed after the initial acceleration would have the same flight time as their precursors.

Another particularly dramatic feature of the ganglioside spectra is the similarity among the sodiated spectra of G_{M1} , G_{D1a} , and G_{T1b} obtained without collisional cooling (Figures 1a, 2a, 3a, 3c). These spectra are virtually identical despite being formed from different gangliosides having 1–3 sialic acids. Thus, ganglioside ions formed from these species produce degenerate spectra with a standard MALDI-FTMS ion source, and the analysis of a mixture of gangliosides without some form of precursor stabilization will be limited from the start by the potential loss of all sialic acid residues.

High pressure collisional cooling generates ions with such low average vibrational energy that noncovalent matrix adduction can be observed. In the spectra shown in Figures 4 and 5, the gangliosides were ionized in the negative ion mode as sodium-exchanged molecular ions. In each spectrum, the base peak is, again, the unfragmented, sodium-exchanged molecular ion, but the addition of up to three non-covalently adducted matrix molecules to the ganglioside is also readily apparent. However, since these ions are trapped and stored in the FTMS, it should be possible to remove these adducts using a low amplitude collisional activation event or a low intensity infrared multiphoton

dissociation (IRMPD) pulse [42]. In Figure 4b the sodiated molecular ion is the base peak, but the peaks lower in mass were not identifiable as fragments from the G_{P1} ganglioside. Instead, those peaks marked with an asterisk were identifiable as ATT matrix clusters. Since this sample was extracted from human brain tissue [37, 38] stored for ten years and was of unknown concentration, it is not surprising that the signal to noise ratio differs from the other spectra of ganglioside standards. Also, oddly, in Figure 5 the doubly matrix adducted peak at m/z 2527 was inexplicably 2.03 Da lower than expected for the simple addition of two molecules of ATT. This unusual adduct formation will be investigated further.

Methods for separation of gangliosides by thin layer chromatography (TLC) are well established [14, 43–45], and previous work in our own and in other laboratories has shown that TLC can be easily coupled to MALDI-TOF MS [31, 46, 47], with some compromise in instrument performance due to the irregular surface [31]. The ability to desorb gangliosides without fragmentation, as shown here, allows coupling TLC separations to MALDI-FTMS while retaining spectral quality. This arrangement will provide the high resolution, high mass accuracy, and tandem mass spectrometry capabilities of the FTMS to characterize species separated by TLC and evaluated for biological activity by overlay assay with specific antibodies or ligands, e.g., microbes. Since TLC assays are one of the most important methods in the field of glycobiology, TLC-MALDI-FTMS is, therefore, the focus of ongoing development in our laboratory.

Conclusion

Transiently elevating pressure in a MALDI-FTMS source into the 1–10 mbar range during ionization decreases the metastable fragmentation of gangliosides. This allows detection of the molecular ion species without loss of the highly labile sialic acid residues. In these experiments, sodiated gangliosides with up to five sialic acids were ionized by MALDI and detected with the FTMS. In each case, when the high pressure collisional cooling was used, the singly charged molecular ion was the base peak in the spectrum and minimal metastable fragmentation was observed. This result is promising as the previously developed TLC separation methods can be coupled to MALDI FTMS.

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